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The Akt/PKB serine/threonine kinase plays roles in coupling growth factor receptors to cell survival pathways. Although evidence suggests roles for phosphatidylinositol 3'-OH kinase (PI3K) and Akt in normal mammary development 1 and tumorigenesis, the role of these signaling molecules in these processes remains to be elucidated . To directly assess the role of Akt in mammary epithelial development and tumorigenesis, we have generated transgenic mice that express a constitutively active Akt (Akt-DD) in the mammary epithelium. Although expression of Akt-DD interferes with normal mammary gland involution, mammary tumors were not observed in these strains. To explore the potential role of Akt in mammary tumor progression, mice co-expressing Akt-DD and a mutant form of Polyomavirus middle T (PyV mT) antigen de-coupled from PI3K/Akt signaling pathways were generated. Co-expression of Akt-DD with mutant PyV mT resulted in dramatic acceleration of mammary tumorigenesis. This acceleration was further correlated with reduced apoptotic cell death in mammary epithelium expressing the mutant form of PyV mT. Taken together these observations indicate that activation of Akt can contribute to tumor progression by providing an important cell survival signal.

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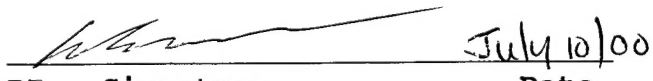

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Introduction

The major focus of DOD sponsored research program is to assess the role of the phosphadityl inositol 3í (PI-3í) and Akt-1 kinases in mammary tumorigenesis and metastasis. Although evidence suggests roles for phosphatidylinositol 3í-OH kinase (PI3K) and Akt in normal mammary development and tumorigenesis (Farrelly et al., 1999; Stambolic et al., 1998; Webster et al., 1998),the role of these signaling molecules in these processes remains to be elucidated . To directly assess the role of Akt and PI-3í kinase in mammary epithelial development and tumorigenesis, we have generated transgenic mice that express a constitutively active Akt (Akt-DD) or PI-3í kinase in the mammary epithelium. To accomplish this goal we have generated transgenic mice that carry constyitutively activated forms of the Akt and PI-3í kinase under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer. To this end we thus far derived 11 independent transgenic lines carrying the MMTV/activated Akt strains and 4 independent lines expressing the MMTV/activated PI-3í kinase. Expression amalyses of these strainshas revealed that 3 of 11 MMTV/activated Akt mice express the activated Akt transgene in the mammary epithelium whereas we have not yet detected transgene expression in any of the MMTV/PI-3í kinase strains. Although expression of Akt-DD interferes with normal mammary gland involution, mammary tumors were not observed in these strains. To explore the potential role of Akt in mammary tumor progression, mice co-expressing AktñDD and a mutant form of Polyomavirus middle T (PyV mT) antigen de-coupled from PI3K/Akt signaling pathways were generated. Co-expression of Akt-DD with mutant PyV mT resulted in dramatic acceleration of mammary tumorigenesis. This acceleration was further correlated with reduced apoptotic cell death in mammary epithelium expressing the mutant form of PyV mT. Taken together these observations indicate that activation of Akt can contribute to tumor progression by providing an important cell survival signal.

Body

Generation of transgenic mice expressing activated versions of the Akt and PI-3í kinase in the mammary epithelium.

To further assess the importance of the PI3K/Akt signaling pathway in PyV mT induced tumorigenesis and metastasis we derived transgenic mice that express a constitutively active version of Akt (HAPKB308D473D or Akt-DD), which mimics the active phosphorylated state of the protein, in the mammary gland (Alessi et al., 1997)(Fig. 1a). To distinguish between the transgene derived and endogenous Akt protein a Haemaglutinin (HA) epitope tag was placed in frame at the amino terminus of the activated Akt protein (Fig 1a). Initially, eleven activated MMTV/Akt founder lines were derived. Nine of these lines passed the transgene to their offspring and a screen for expression of the activated Akt transgene revealed expression in the mammary gland in three of these lines (Table 1). The tissue specificity of transgene protein product expression of two of these lines (MMTV/Akt7 & MMTV/Akt10) was determined and the higher expresser (MMTV/Akt7) was chosen for further study (Table 1). To confirm that activated Akt protein product was expressed in the mammary epithelium of transgenic

mice, multiple mammary tissue extracts from the Akt-7 line were subjected to anti-HA immunoblot analysis. The results revealed that virgin mammary glands from these strains were expressing significant levels of the transgene-derived Akt protein (Fig. 1b). In contrast to our experience with MMTV/activated Akt atrains RNase protection analyses with a transgene specific probe of 3 of 4 transgenic strains has yet to reveal the presence of transgene expressing strain (Table 1, Appendix 1). We will shortly be examining the final MMTV/PI-3' kinase strain (PI3K3 strain, Table 1) for mammary gland specific expression of the transgene. Given the lack MMTV/PI-3' kinase expressing strains we are planning to derive further founder strains with this construct.

Mammary epithelial expression of activated Akt results in defects in mammary gland involution.

To ascertain whether elevated expression of activated Akt could interfere with normal mammary gland development, wholemount analyses of both virgin and involuting mammary glands were conducted. Virgin female glands from MMTV/Akt strains were histologically and morphologically identical to FVB/N female controls (data not shown). Consistent with these observations, female Akt transgenic mice have yet to develop mammary tumors after a year of observation. Given the importance of apoptotic cell death in mammary gland involution, we next examined whether mammary gland involution was adversely affected in the activated Akt strains. To explore this possibility, mammary glands from wild type and activated Akt strains were examined at 1, 3 and 7-days of involution. In contrast to wild type control animals which exhibited extensive involution at 1 and 3-days post-involution (Fig. 2 a, b, e, f), the Akt animals displayed a dramatic defect in mammary gland involution (Fig.2c,d,g,h). However, the Akt mammary glands eventually underwent full involution at 7-days post-involution (Fig 2i,j,k,l) due to a drop in MMTV driven transgene expression in the activated Akt strains. A similar involution defect was noted in the Akt-10 strain indicating that this phenotype was not dependent on the transgene integration site.

To explore whether the observed involution defect was due to the ability of activated Akt to interfere with the levels of apoptotic death, we measured the levels of in situ apoptotic death using TUNEL analyses. Consistent with the wholemount analyses, the results revealed that MMTV/activated Akt strains exhibited decreased numbers of apoptotic cells at days 1 and 3 of involution compared with wild type siblings (Fig. 3, Appendix 1). Taken together, these observations argue that activation of Akt can interfere with normal mammary gland involution by reducing the levels of apoptoc cell death in the mammary epithelium.

Activation of Akt/PKB in mammary epithelium provides a critical cell survival required for tumor progression.

Although these data suggest that the Akt-DD mutant can interfere with apoptotic cell death during mammary gland involution, its role in mammary tumorigenesis is unclear. To explore whether active Akt expression could complement the defect in tumorigenesis exhibited by transgenic mice expressing the mutant PyV mT de-coupled from the PI3K/Akt signaling pathway (Webster et al., 1998), bi-transgenics expressing

both the Akt transgene and the mutant MT transgene (MTY315/322F) were derived. To confirm that both mutant PyV mT and Akt proteins were expressed we performed immunoblot analyses with antibodies directed to Akt or PyV mT proteins. The results of these immunoblot analyses revealed that both Akt and mutant PyV mT proteins were efficiently expressed in the mammary tissues (Fig 4a, Appendix 1).

To determine whether co-expression of activated Akt and mutant PyV mT oncogenes resulted in in acceleration of mammary tumor onset, two independent cohorts of virgin female mice were subjected to physical palpation. The results of these analyses revealed that bi-transgenic mice developed mammary tumors with 100% penetrance with an average latency of 46 and 54 days respectively (Fig. 4b). In contrast physical palpation of two independent cohorts of female mice carrying the mutant PyV mT transgene alone revealed a significant delay in the onset of tumor formation (T50 of 123 and 119 days respectively) (Fig. 4b). Consistent with these kinetic analyses, wholemount analyses of virgin mammary glands of bi-transgenic mice revealed a dramatic difference in the extent of tumor growth (compare Fig. 5g,h to 5e,f). In contrast to the diffuse cystic hyperplasias exhibited by the mutant PyV mT strains , female transgenic mice co-expressing the mutant PyV mT and activated Akt transgenes exhibited differentiated carcinomas. Consistent with these analyses these lesions could be subcutaneously transplanted into syngeneic recipients. Because the mammary epithelial hyperplasias associated with the mutant PyV mT strains possess elevated levels of apoptotic cell death, we measured the degree of apoptotic cell death in mammary glands derived from the mutant PyV mT or bi-transgenic mice. The results revealed that mammary epithelial expression of activated Akt resulted in a dramatic repression of the high rates of apoptotic cell death in PyV mT mutant tissue de-coupled from the PI3K (Fig. 6). Taken together, these observations argue that the dramatic acceleration of mammary tumorigenesis exhibited by these strains is due to the ability of activated Akt to suppress the elevated apoptotic cell death displayed by mutant PyV mT mammary epithelium.

Although the active, transgenic Akt is able to complement the mutant PyV mT strains for the induction of mammary tumors only 20% of the tumor bearing mice have developed lung metastases more than 8 weeks after the initial palpation of the mammary tumor (n=10). The penetrance of the metastatic phenotype is comparable to the 30% metastasis levels exhibited by the parental mutant PyV strains. In contrast, 67% of mice expressing wildtype MT show multiple lung metastases at comparable time points and tumor loads (Fig. 4c). These observations argue that while expression of active Akt can complement the defect in mammary tumor progression, it is unable to rescue the defect in metastatic progression.

Key Research Accomplishments

- **Generation of both MMTV/PI-3' kinase and MMTV/activated Akt transgnic strains.**
- **Demonstrated that mammary epithelial expression of activated Akt results in a delay of mammary involution dsue to supression of apoptotic cell death..**
- **Demonstrated that coexpression of activated Akt and mutant PyV mT oncogene decoupled from the PI-3' kinase can result in dramatic acceleration of tumor onset due to supression of apoptotic cell death.**

Reportable Outcomes

Presentation at the 16 th annual Meeting on Oncogenes
J.Hutchinson, J, Jing, R. Cardiff, J. Woodget and W.J. Muller
Mammary epithelial expresssion of Akt/PKB affects mammary gland involution and tumor progression

Conclusions

The studies outlined above provide compelling evidence that expression of activated Akt is involved in promoting tumor progression by providing a critical cell survival pathway. Consistent with this contention, mammary epithelial expression of Akt can result in profound delays in mammary gland involution, a process involving extensive apoptotic cell death. Moreover, co-expression of activated Akt can suppress the elevated rates of apoptotic cell death that are observed in mammary epithelial hyperplasias induced by the mutant PyV mT de-coupled from the PI3K signaling pathway. However, because mammary epithelial expression of Akt does not result in the induction of mammary tumors itself, tumorigenesis requires the constitutive activation of other signaling pathways that are recruited by the mutant PyV mT oncogene including the Src family kinases and Shc/Grb2/Ras pathway. Although our studies suggest that activated Akt can cooperate with these signaling pathways to efficiently induce mammary tumorigenesis, the observed low rates of metastasis suggests the involvement of other PI3K dependent signaling pathways in the potent metastatic phenotype exhibited by wild type PyV mT. For example, PI3K activation can modulate the activity of members of the Rho family of GTP-binding proteins (Rodriguez-Viciana et al., 1997) and the Integrin-Linked Kinase (ILK) (Delcommenne et al., 1998). In this regard, the roles of these sets of signaling molecules in cell migration and adhesion implicates them in metastatic progression (Dedhar et al., 1999). Further crosses with MMTV/activated PI-3' kinase strains should allow us to address the importance of these PI-3' kinase dependent pathways.

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Farrelly, N., Lee, Y. J., Oliver, J., Dive, C. and Streuli, C. H. (1999). *J Cell Biol* **144**, 1337-48.

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Table 1. Transgene Expression in MMTV/Akt and MMTV/PI3K mice[a]

	Expression of transgene[b] in:												
	Female										Male		
Line	MGl	Brain	Heart	Kidney	Liver	Lung	Ovary	Salivary	Spleen	Thymus	Epidid	SemVes	Testes
Akt1	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt2	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt3	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt4	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt5[c]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt6	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt7	++	-	-	-	-	-	-	-	-	-	++++	++	-
Akt8	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt9	+	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Akt10	+	-	-	-	-	-	-	-	-	-	+++	++++	-
Akt11[c]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
PI3K1	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
PI3K2	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
PI3K3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
PI3K4	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

[a] Expression of the Akt and PI3K transgenes in the mammary gland was initially determined via RNase protection analysis on 20 μg of total RNA with a probe directed against the SV40 polyA region of the transgene. Subsequently, expression of the Akt transgene in the mammary gland and other tissues of expressors was determined by Western blot analysis using the HA-11 monoclonal antibody (Babco) on 250 μg of total protein lysate pre-cleared in ProteinG-Sepharose.

[b] Relative levels of transgene expression: nd, no data; -, not detected; +, low; ++, intermediate; +++, high; ++++, very high; MGl, normal mammary gland; Epidid, epididymus; SemVes, seminal vesicles.

[c] Strain did not pass transgene.

Figure 1 – Activated Akt cDNA construct and tissue specific expression in MMTV/Akt transgenic mice.

(a) Structure of the MMTV/Akt transgene. The Bluescript vector backbone is represented by a thin line on either side of the expression cassette, with the white region corresponding to the MMTV LTR derived from plasmid pAp, the green portion corresponding to the hemaglutinin tag, the yellow region corresponding to the Akt (HAPKBT308D/S473D) cDNA with aspartate substitutions at amino acid positions 308 and 473, and the blue region corresponding to the transcriptional processing sequences derived from the SV40 early transcription unit. The transcription start site is indicated by the arrow.

(b) Transgene expression in MMTV/Akt transgenic mice. Protein corresponding to the MMTV/Akt transgene in various organs of the Akt7 and Akt10 transgenic strains as assessed by Western blot against the HA tag (Babco HA-11) on two-hundred and fifty micrograms of total protein lysate pre-cleared in ProteinG-Sepharose. Tissues were derived from 8 week old virgin females and males. Also shown are control Grb2 Western blots on matched protein samples. M.Gl., mammary gland; Epidid., epididymus; Sem.Ves., seminal vesicles.

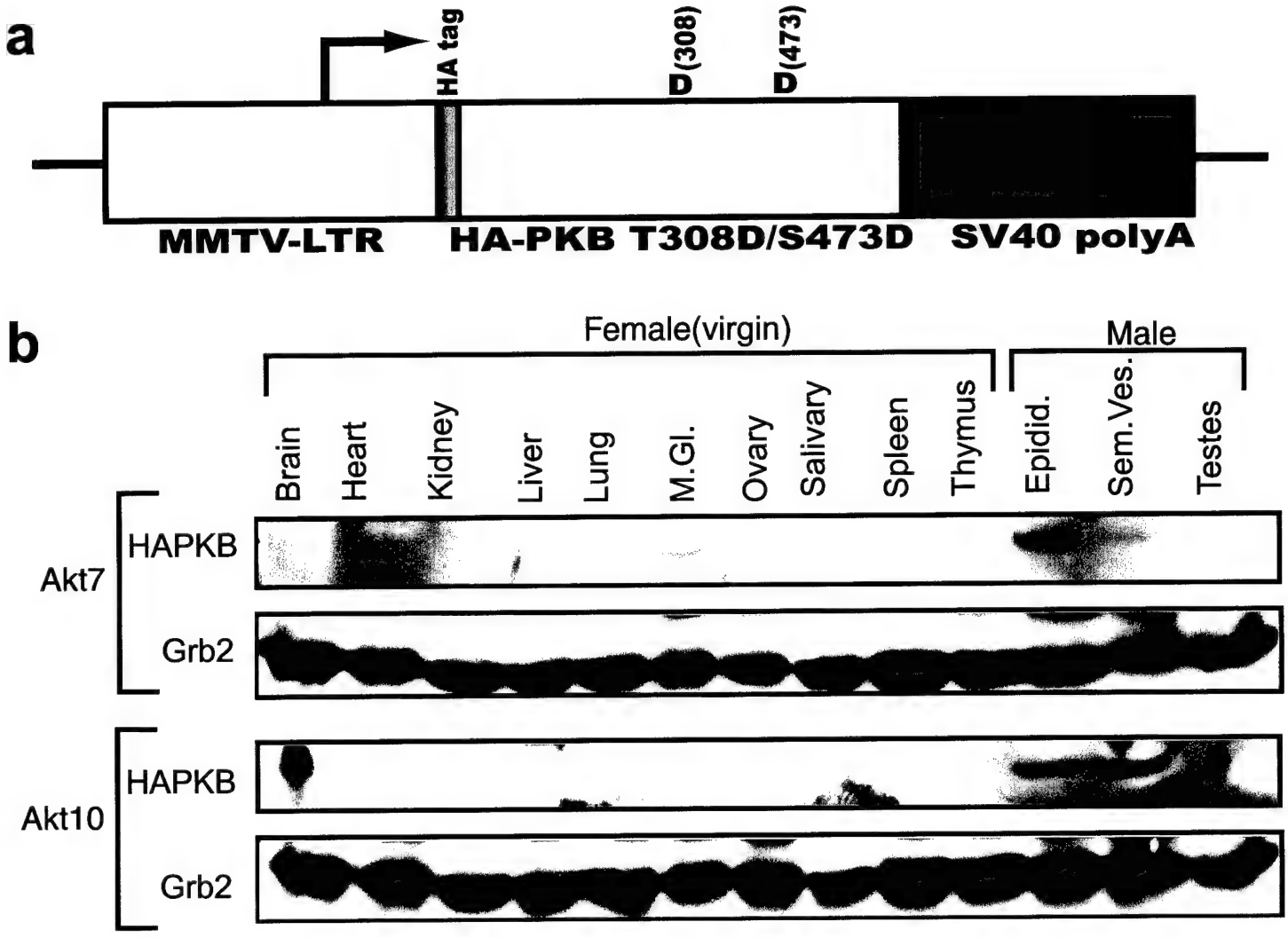

a
HA tag
D(308)
D(473)
MMTV-LTR
HA-PKB T308D/S473D
SV40 polyA
b
Female(virgin)
Male
Brain
Heart
Kidney
Liver
Lung
M.Gl.
Ovary
Salivary
Spleen
Thymus
Epidid.
Sem.Ves.
Testes
Akt7
HAPKB
Grb2
Akt10
HAPKB
Grb2

Figure 2 – Mammary epithelial expression of Akt results in defects in mammary gland involution.

Digital images of the involution patterns in wild type (**a,b,e,f,l,j**) and activated Akt strain (**c,d,g,h,k,l**). The images compare whole mount preparations (**b,d,f,h,j,l**) with histological patterns (**a,c,e,g,l,k**) on days 1 (**a-d**), 3 (**e-h**) and 7 (**i-l**) of involution. Note the delayed involution in the Akt mouse mammary gland (**c,d,g,h,k,l**).

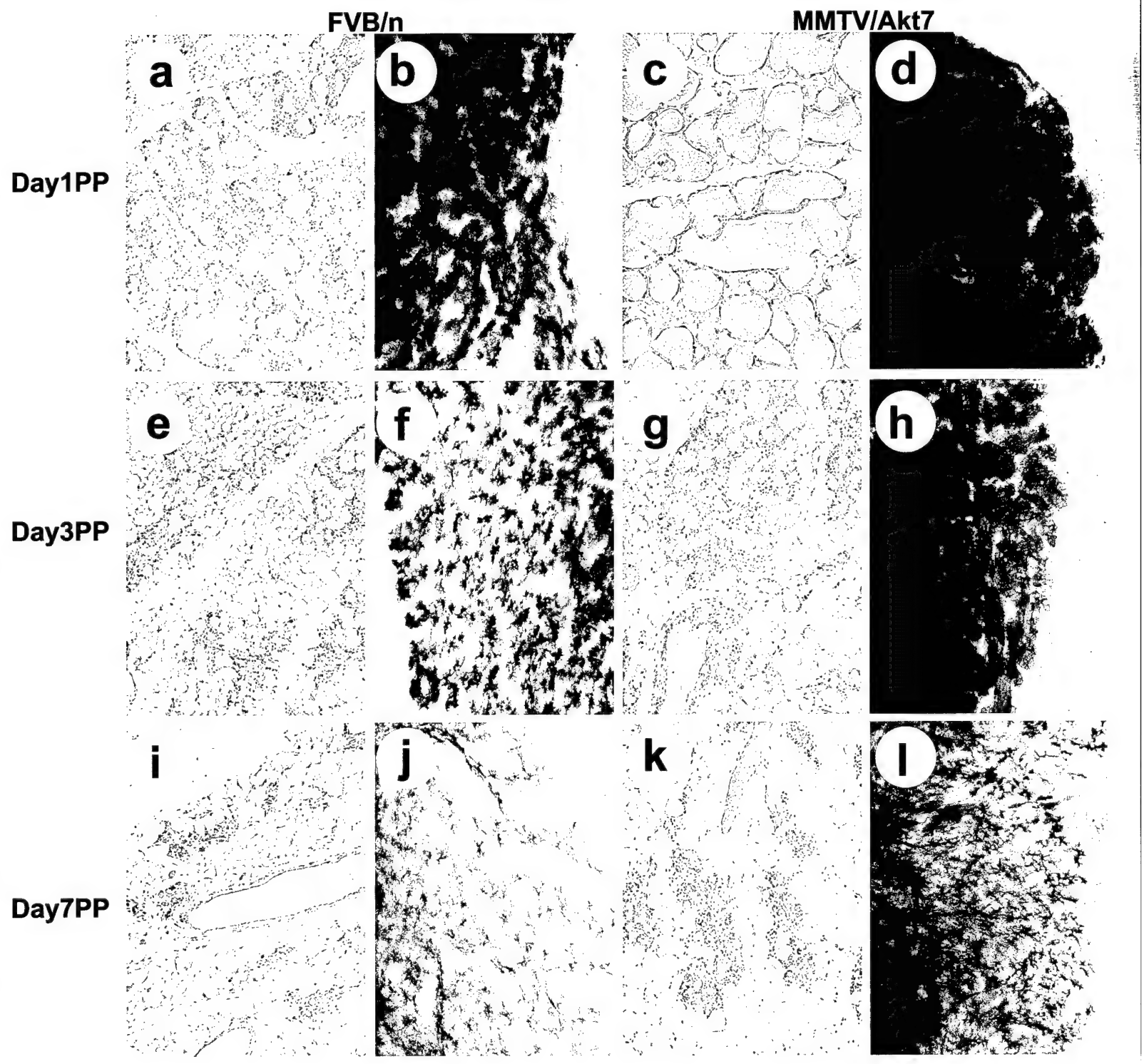

FVB/n
MMTV/Akt7
Day1PP
Day3PP
Day7PP
a
b
c
d
e
f
g
h
i
j
k
l

Figure 3 – Mammary epithelial expression of Akt reduces apoptosis during mammary gland involution.

Apoptotic indices of involuting mammary glands in FVB and Akt strain. Values shown represent the percentage of total cells positive for apoptosis by TUNEL assay in singly parous female mice.

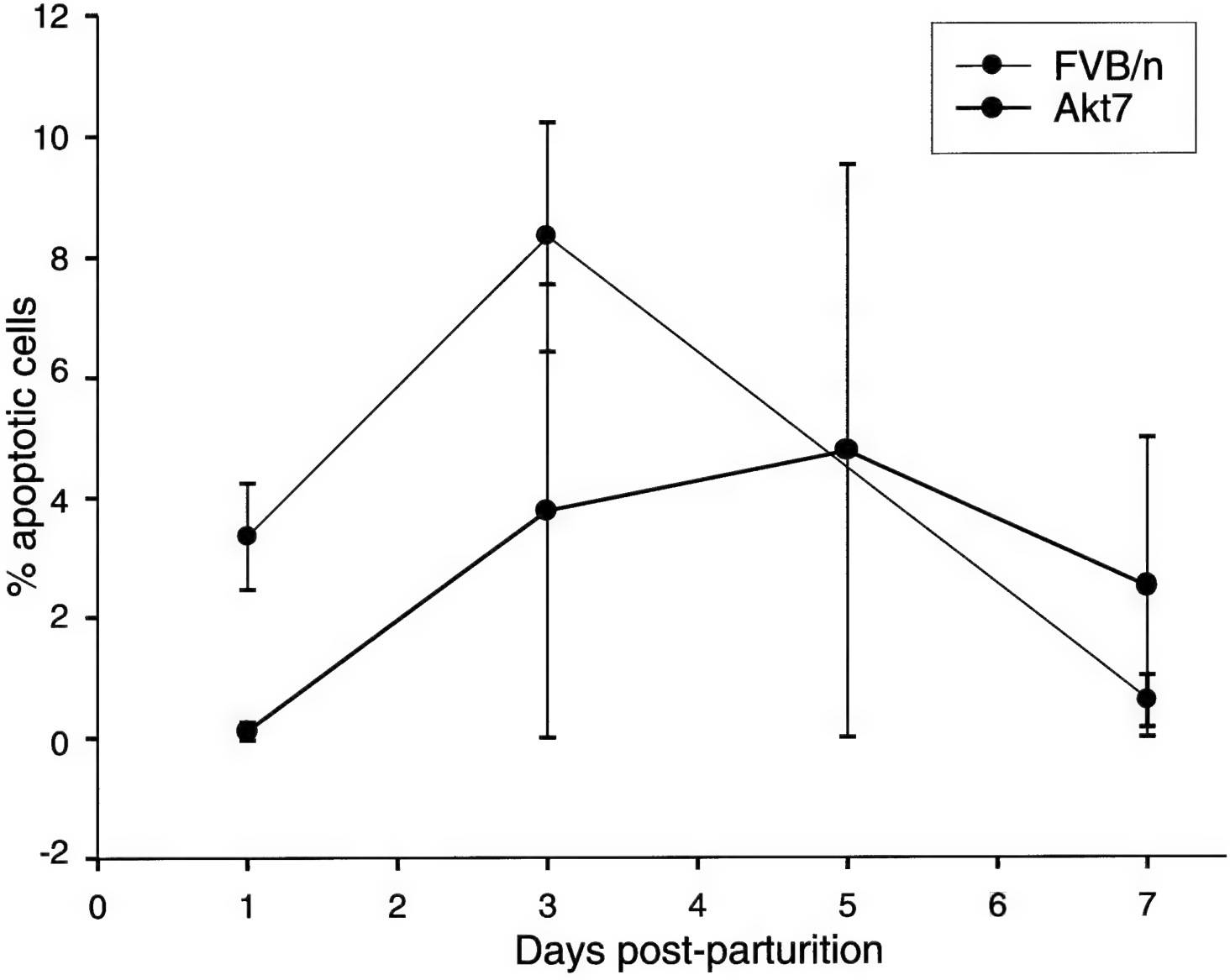

12
10
8
6
4
2
0
-2
% apoptotic cells
0
1
2
3
4
5
6
7
Days post-parturition
FVB/n
Akt7

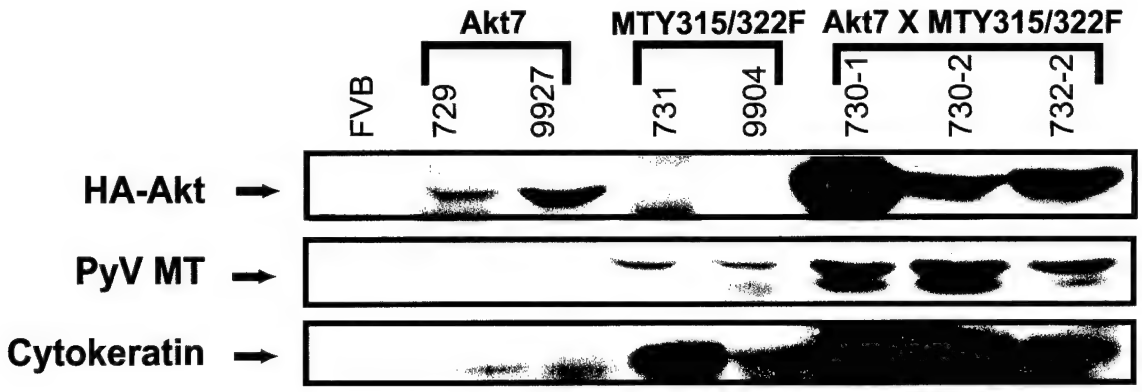
Figure 4 – Co-expresion of Akt and mutant PyV mT oncogene results in the induction of multifocal mammary tumors.

(a) Immunoblot analyses of mammary tissue from the FVB, Akt7, MTY315/322F and Akt7 X MTY315/322F strains. Total protein lysate was subjected to anti-HA immunoblot analysis with HA-11 monoclonal antibody (Babco). PyV MT was immunoprecipitated from total protein lysate with Pab762 (courtesy Dr. S. Dilworth) and subjected to anti-MT immunoblot analysis with Pab701 (courtesy Dr. S. Dilworth). Anti-cytokeratin immunoblot analysis was carried out on total protein lysate using Troma-1 rat monoclonal antibody. Higher levels of Akt and MT may reflect higher epithelial cell content of tissue samples as revealed by anti-cytokeratin analysis.

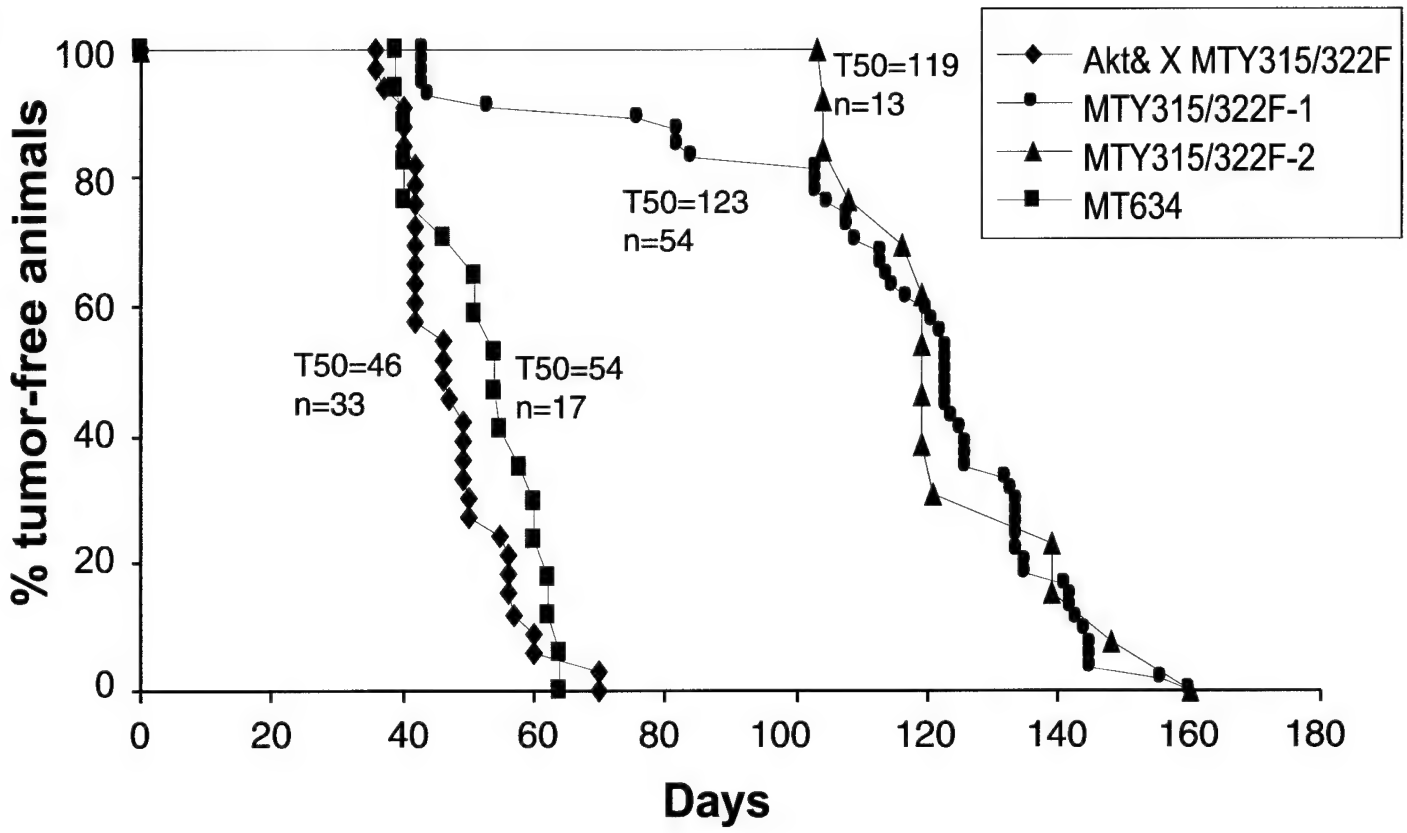
(b) Mammary tumor kinetics in transgenic strains. Mammary tumor kinetics of MTY315/322F and Akt7XMTY315/322F strains. Two different kinetics curves are shown for the MTY315/322F strain, the original data (MTY315/322F-1) and confirmatory data by the current researcher (MTY315/322F-2) to account for possible differences in palpation technique. Age indicated is that at which a mammary tumor is first palpable in each transgenic strain. The number of animals analyzed for each strain (n) and the median age at which tumors are palpable is shown.

(c) Lung metastases were assessed by histological analysis of lung tissue of animals 30 or more days after palpation of the initial tumor.

a



b



c

Transgenic Line	# Mice	Lung Metastases	% Metastasis
MT634	9	6	67
MTY315/322F	42	15	36
Akt X MTY315/322F	10	2	20

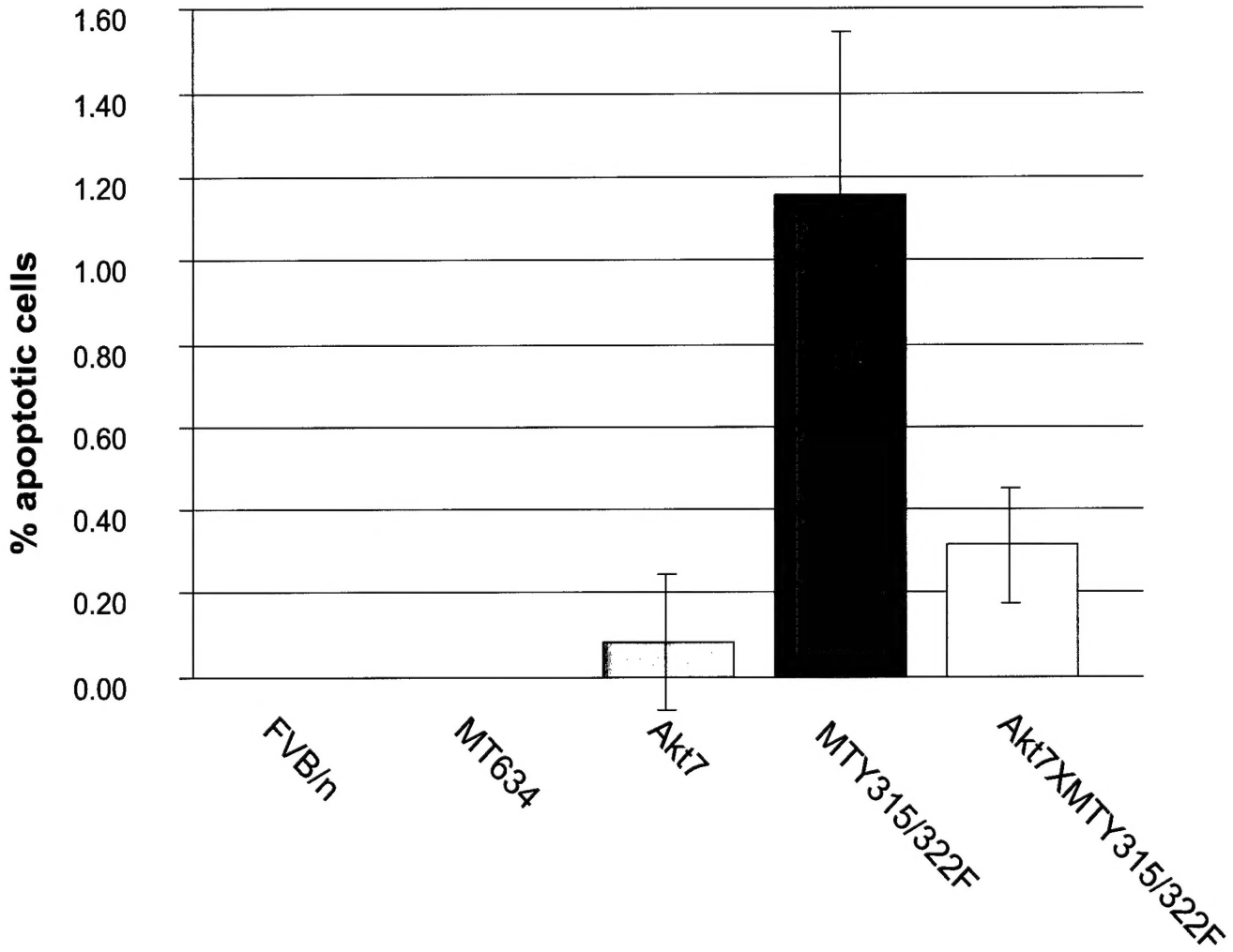
Figure 5 – Histological analysis of mammary epithelium of transgenic mice expressing activated Akt and mutant PyV mT oncogene.

These digital images illustrate the histological patterns observed in wiltype FVB (**a,b**), the Akt7 (**c,d**) MTY315/322F (**e,f**) and Akt7 X MTY315/322F bigenic mice (**g,h**). Note that the wholemount preparations (**a,c,e,g**) demonstrate that the Akt strains have a relatively normal mammary tree (**a,c**) compared to the cystic hyperplasias seen in the MTY315/322F strains at the same age (**e**) (6.5 weeks). In contrast the bigenic mammary gland is a solid mass at this age (**g**). The histological patterns seen at high magnification demonstrate that the Akt7 strain has a normal epithelium (**b,d**), while the MTY315/322F has a cystic hyperplasia of the ducts and glands without significant atypia (**f**). In contrast, the Akt7 X MTY315/322F cross has acinar or lobular hyperplasia with low grade atypia at eight weeks (**h**).

FVB/n
Akt7
MTY315/322F
Akt7XMTY315/322F
a
b
c
d
e
f
g
h

Figure 6 – Mammary epithelial expression of Akt decreases apoptosis in MTY315/322F mammary glands.

Mammary apoptotic indices of mammary glands patterns in wild type, Akt, AKt7XMTY315/322F and wildtype MT634 strains. Values shown represent the percentage of total cells stained positive for apoptosis by TUNEL assay in virgin female mice at 8-10 weeks of age.


% apoptotic cells
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
FVB/n
MT634
Akt7
MTY315/322F
Akt7XMTY315/322F